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Diacyl Choline Phosphoglyceride: The Endogenous Substrate for Energy Metabolism in Sea Urchin Spermatozoa

Masatoshi Mita

Department of Biochemistry, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173, Japan

ABSTRACT—Endogenous choline phosphoglycerides (CPG) are substrates for energy metabolism in the spermatozoa of the sea urchin, *Hemicentrotus pulcherrimus*. It has also been reported that alkenylacyl, alkylacyl and diacyl phosphoglycerides are distributed in sea urchin spermatozoa. This study was undertaken to determine whether CPG available for utilization in energy metabolism is a diacyl and/or ether-containing compound. After incubation of spermatozoa in seawater, only the diacyl choline CPG content was found to have decreased significantly, and no changes were detectable in the other phospholipids. Analysis by gas-liquid chromatography indicated that 16:0, 18:0, 20:1, 20:4 and 20:5 at the 1-position and 20:4 and 20:5 at the 2-position of diacyl CPG had decreased during incubation. Phospholipase A_2 activity also had high substrate specificity for diacyl CPG. Thus it seems likely that sea urchin spermatozoa obtain energy throught the oxidation of diacyl CPG.

INTRODUCTION

Flagellar movement in sea urchin spermatozoa occurs partly through reactions catalyzed by dynein ATPase [1–4]. Thus, energy metabolism for production of ATP is indispensable for swimming. Sea urchin spermatozoa have been shown to obtain energy for movement from oxidation of endogenous phospholipid [5–7]. It has also been reported that sea urchin spermatozoa contain various phospholipids and cholesterol [8, 9]. Triacylglycerol and glycogen are also present in trace amounts [7–9]. Choline, ethanolamine and serine phosphoglycerides (CPG, EPG and SPG) are the predominant components. After incubation of spermatozoa in seawater, the content of endogenous CPG has been shown to be decreased significantly, with no change in the levels of other phospholipids [8, 10]. CPG thus appears to be a substrate for energy metabolism in sea urchin spermatozoa. The preferential hydrolysis of CPG, among the phospholipids available for energy metabolism, is related to the properties of phospholipase A_2, which in sea urchin spermatozoa has high substrate specificity for CPG [10].

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In addition to diacyl phospholipids, ether-containing derivatives, such as alkenylacyl EPG, plasmalogen, have been shown to be present in sea urchin spermatozoa [11, 12]. It was shown recently that CPG contained alkylacyl (19%) and diacyl (81%) components [13]. However, previous studies [8, 10] on energy metabolism have been done using mixtures of diacyl and ether-containing phosphoglycerides. Thus, whether the CPG available for utilization in energy metabolism is a diacyl and/or ether containing compound is still unclear. For further clarification of energy metabolism using phospholipids, alkenylacyl, alkylacyl and diacyl phospholipids in spermatozoa of *Hemicentrotus pulcherrimus* were analyzed in this study.

MATERIALS AND METHODS

Materials

Spermatozoa of the sea urchin, *H. pulcherrimus*, were obtained by forced spawning induced by injection of 0.5 M KCl into the coelomic cavity. Semen was always collected freshly as 'dry sperm' and kept undiluted on ice. The number of spermatozoa was calculated on the basis of protein concentration, as determined by the method of Lowry *et al.* [14], using bovine serum albumin as the

standard. The protein content per 10^9 spermatozoa was 0.50 mg.

Extraction of lipids

Dry sperm were diluted 100-fold in artificial seawater (ASW) containing 458 mM NaCl, 9.6 mM KCl, 10 mM $CaCl_2$, 49 mM $MgSO_4$ and 10 mM Tris-HCl at pH 8.2, and incubated for 1 hr at 20°C. Each sample was centrifuged at $3,000 \times g$ for 5 min at 0°C. Total lipids were extracted from the precipitate by the method of Bligh and Dyer [15]. Individual phospholipids were separated by thin-layer chromatography (TLC) as described previously [8, 9].

Separation of alkenylacyl, alkylacyl and diacyl phospholipids

Alkenylacyl, alkylacyl and diacyl choline, ethanolamine and serine phosphoglycerides were separated as 1,2-diradyl-3-acetylglycerol derivatives as described previously [16, 17]. The contents of each type of 1,2-diradyl-3-acetylglycerol were estimated by gas-liquid chromatography (GLC) assays of amounts of fatty acyl moieties in the lipid class, using 17:0 methyl ester as the internal standard [16].

Determination of fatty acid composition

The fatty acyl residues of diacyl CPG were analyzed as the methyl esters by GLC [8, 9]. To investigate the positional distribution of fatty acids in 1,2-diacyl-3-acetylglycerol, fatty acids at the 1-position were liberated by *Rhizopus delemain* lipase (Sigma) and the resulting monoglycerides were separated by TLC and transmethylated as described previously [16, 18]. The fatty acid methyl-esters were extracted with *n*-hexane, followed by N_2-blow evaporation. The residues were dissolved in a small amount of *n*-hexane and analyzed using a GC-R1A gas-liquid chromatograph (Shimadzu, Kyoto) equipped with a coiled column packed with 15% EGSS-X.

Estimation of phopholipase A_2 activity

Dry sperm were homogenized with 10 mM $MgCl_2$, 10 mM $CaCl_2$, 1 mM dithiothreitol and 50 mM Tris-HCl at pH 7.5. The homogenate was incubated with either 4.6 kBq 1-palmitoyl-2-[1-^{14}C]-arachidonyl-CPG(2.18 GBq/mmol)(Du Pont-New England Nuclear) or 18.5 kBq 1-*O*-hexadecyl-2-[5,6,8,11,12,14,15-^{3}H(N)]-arachidonyl-CPG (2749.1 GBq/mmol) (Du Pont-New England Nuclear) for 1 hr at 20°C in a total volume of 0.4 ml, followed by extraction of total lipids. Radioactivity in the free fatty acid fraction separated by TLC was measured by liquid scintillation spectrometry.

RESULTS

CPG, EPG and SPG extracted from sea urchin spermatozoa have been shown to have an ether-containing component in addition to the diacyl

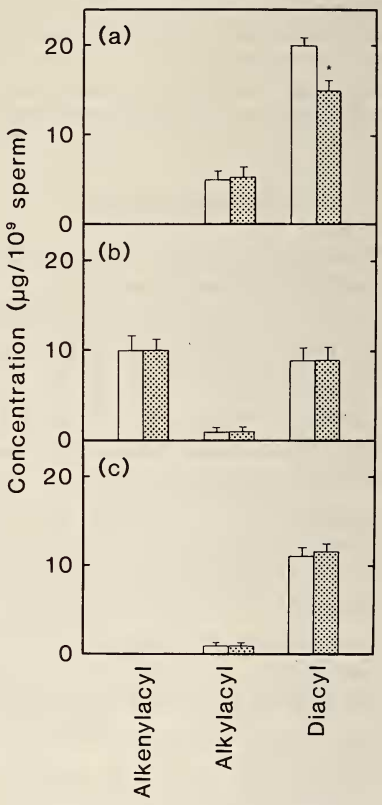

FIG. 1. Changes in levels of CPG (a), EPG (b) and SPG (c) in sea urchin spermatozoa following incubation with seawater. Dry sperm were diluted 100-fold in seawater and incubated for 1 hr at 20°C. Before (clear) and after (dotted) incubation, total lipids were extracted from spermatozoa. CPG, EPG and SPG separated by a thin-layer chromatography were used for analysis of alkenylacyl, alkylacyl and diacyl derivatives. Each value is the mean of three separate experiments. Vertical bars show S.E.M.

component [13]. An experiment was carried out to confirm this, and to determine whether the contents of the alkenyl-acyl, alkylacyl and diacyl components of CPG, EPG and SPG decreased during incubation in seawater. Before incubation, CPG contained alkylacyl ether (19%) and diacyl (81%) components (Fig. 1). A trace amount of alkenyl-acyl analog was also present. EPG consisted of 47% alkenyl ether, 2% alkyl ether and 51% diacyl compounds. The SPG fraction contained a considerable amount of the diacyl compound (91%). The content of the alkyl ether compound was only 9%, and the alkenyl ether analog was present only at a trace level. After incubation of sea urchin spermatozoa for 1 hr at 20°C, the preparation of alkylacyl CPG had increased from 19% to 27%, and diacyl CPG had decreased from 81% to 73%. The level of the CPG mixture in dry sperm (25 μg/10^9 spermatozoa) thus decreased (20 μg/10^9 spermatozoa) following incubation. Similarly, the net content of diacyl CPG decreased from 20 ± 1 μg/10^9 spermatozoa to 15 ± 1 μg/10^9 spermatozoa (Fig. 1). However, the alkyl ether CPG content was almost constant (5 ± 1 μg/10^9 spermatozoa) during incubation.

Previous studies have shown that CPG, composed partly of unsaturated fatty acids, is consumed preferentially during incubation [8, 9, 19]. The fatty chain moieties at the 1- and 2-positions in diacyl CPG metabolized during incubation were also examined. Fatty acids at the 1-position of diacyl CPG were mostly of the saturated and monoenoic type, such as 16:0 (22%) and 20:1 (19%) (Table 1). 20:4 (13%) was also present at the 1-position. By contrast, significant amounts of 20:4 (47%) and 20:5 (23%) were found primarily among fatty acids at the 2-position. During dilution and incubation in ASW for 1 hr at 20°C, the relative percentages of fatty acids at the 1- and 2-positions of diacyl CPG remained almost constant (Table 1).

Based on the net content of diacyl CPG shown in Fig. 1, changes in the levels of fatty acid moieties at the 1- and 2-positions in diacyl CPG

Table 1. Fatty acid composition of diacyl choline phosphoglyceride in sea urchin spermatozoa before and after incubation in seawater

Fatty acid	Dry sperm		Incubation for 1 hr	
	1-position	2-position	1-position	2-position
14:0	1.8±0.2	tr.	2.4±0.4	tr.
15:0	0.7±0.1	tr.	0.6±0.1	n.d.
16:0	22.1±0.2	2.5±0.1	23.2±0.7	2.8±0.2
16:1	3.5±0.1	2.5±0.1	3.9±0.1	2.9±0.3
18:0	3.3±0.3	0.2±0.1	4.1±0.1	0.2±0.1
18:1	11.6±0.4	3.9±0.1	11.2±0.1	3.6±0.2
18:2	1.1±0.1	1.0±0.1	1.0±0.1	1.0±0.1
18:3	0.3±0.1	n.d.	0.5±0.1	n.d.
18:4	6.3±0.3	10.2±0.9	6.4±1.0	10.3±1.8
20:1	19.1±0.8	2.3±0.1	17.4±1.0	2.2±0.1
20:2	3.8±0.6	n.d.	3.2±0.2	n.d.
20:3	2.4±0.4	5.2±0.6	2.0±0.1	5.7±0.8
20:4 (n-6)	13.0±0.8	46.9±1.2	13.0±0.6	46.8±1.4
20:5 (n-3)	9.3±0.6	22.9±0.9	9.2±0.8	22.2±1.5
22:4	0.8±0.3	1.0±0.3	1.0±0.1	0.7±0.2
22:5	0.4±0.1	tr.	0.3±0.2	0.2±0.1
22:6	0.5±0.1	1.4±0.4	0.6±0.1	1.4±0.3

Dry sperm were diluted 100-fold in seawater and incubated for 1 hr at 20°C. Each value is the percentage of the total and the mean±S.E.M. of three separate experiments. tr., trace amount (less than 0.1%); n.d., not detectable.

during incubation for 1 hr were calculated. 16:0, 18:1, 20:1, 20:4 and 20:5 at the 1-position and 20:4 and 20:5 at the 2-position were decreased (Fig. 2). During incubation for 1 hr, about 7 nmol of fatty acids per 10^9 spermatozoa was liberated from the 1- and 2-positions of diacyl CPG, respectively. In contrast to CPG, no change could be detected in the compositions of EPG and SPG in each lipid class.

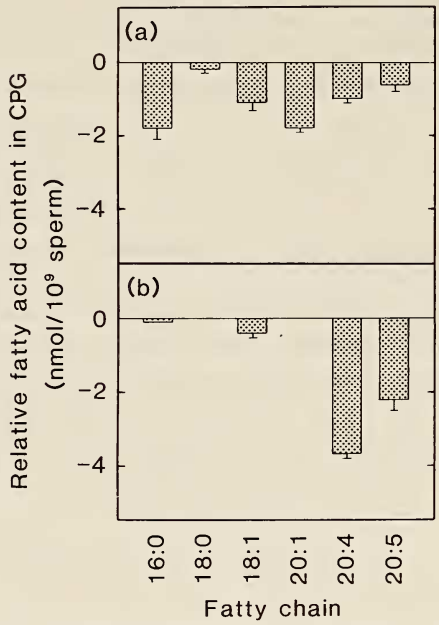

FIG. 2. Changes in levels of fatty acid moiety at the 1- (a) and 2-positions (b) of diacyl CPG following incubation with seawater. Content of fatty acids in diacyl CPG was calculated from the absolute value and the relative percentage of its fatty acid moiety. Each value is the mean of three separate experiments. Vertical bars show S.E.M.

Since the hydrolysis of CPG was shown previously to occur via the action of phospholipase A_2 [8, 10], an examination was conducted to determine whether the phospholipase A_2 is capable of catalyzing alkylacyl CPG in addition to diacyl CPG. The homogenate from dry sperm was incubated with 1-*O*-hexadecyl-2-[5,6,8,11,12,14,15-^{3}H(N)]-arachidonyl-CPG and 1-palmitoyl-2-[1-^{14}C]-arachidonyl-CPG for 1 hr, followed by extraction and separation of free fatty acid by TLC. The radioactivities of ^{3}H- and ^{14}C-free fatty acids were calculated after hydrolysis of alkyl ether and diacyl CPG, respectively. The hydrolysis of alkylacyl CPG was only one tenth of that of diacyl CPG (Table 2). Phospholipase A_2 thus appears to have greater substrate specificity for diacyl CPG.

TABLE 2. Phospholipase A_2 activity in sea urchin spermatozoa

Substrate	Specific activity (nmol CPG hydrolyzed /hr per mg protein)
1-*O*-Alkyl-2-acyl-CPG	0.9±0.2
1,2-Diacyl-CPG	8.8±1.4

The homogenate of dry sperm was incubated with 1-*O*-hexadecyl-2-[5,6,8,11,12,14,15-^{3}H(N)] arachidonyl-CPG or 1-palmitoyl-2-[1-^{14}C]arachidonyl-CPG for 1 hr at 20°C. Each value is the mean±S.E.M. obtained in four separate experiments.

DISCUSSION

The results presented above are further evidence of our previous proposal [8] that CPG is consumed during incubation to provide energy for flagellar movement in sea urchin spermatozoa. CPG available for utilization in energy metabolism was found here to be composed of diacyl compounds (Fig. 1). This preferential hydrolysis of diacyl CPG is due to the particular properties of phospholipase A_2. It was also found that phospholipase A_2 in sea urchin spermatozoa possesses greater substrate specificity for diacyl CPG (Table 2). This appears to confirm the specific use of diacyl CPG for energy metabolism.

In this study, about 5 μg of diacyl CPG was consumed in 10^9 spermatozoa following incubation for 1 hr (Fig. 1). The fatty acids of the consumed diacyl CPG increased 16:0, 18:1, 20:1, 20:4 and 20:5 at the 1-position and 20:4 and 20:5 at the 2-position (Fig. 2). This observation is consistent with data obtained using a mixture of diacyl and ether-containing CPG in previous reports [8, 19, 20]. Previous studies also indicated that ^{14}C-labeled diacyl CPG and fatty acid are oxidized to $^{14}CO_2$ in a cell-free system of sea urchin spermatozoa [8, 10]. Thus possibly, the fatty acid obtained by hydrolysis of diacyl CPG is metabolized for ATP production through β-oxidaton.

In ram spermatozoa, the choline-containing plasmalogen, alkenylacyl CPG, has been shown to be a predominant lipid [21], which is metabolized during aerobic incubation [22]. However, the plasmalogen content of sea urchin spermatozoa remains constant during incubation [11], as confirmed by the present data. Alkenylacyl CPG in sea urchin spermatozoa has been shown to be present in a trace amount [13]. Although a considerable amount of EPG as an alkenylacyl derivative was observed, the levels of alkenylacyl EPG were certainly unchanged after incubation (Fig. 1). Thus, it is unlikely that sea urchin spermatozoa use ether-containing phospholipids as a substrate for energy metabolism. Alkylacyl CPG is well known to be related to the production of a platelet-activating factor (PAF) in leukocytes [23, 24]. Therefore it is of interest that CPG in sea urchin spermatozoa contains an alkylacyl component in addition to the diacyl component [13] (Fig. 1). However, it is unclear whether alkylacyl CPG serves as a PAF precursor in sea urchin spermatozoa.

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